

An Evaluation of the Potential for Hybridization Between *Castilleja levisecta* and *C. hispida*

Thomas N. Kaye and Matt Blakeley-Smith
Institute for Applied Ecology
Corvallis, Oregon

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Washington Department of Natural Resources, Olympia, Washington and
Institute for Applied Ecology, Corvallis, Oregon

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Cover photo: *Castilleja levisecta* and *C. hispida* grown in a greenhouse. Cover photo and other photos by Tom Kaye, Institute for Applied Ecology, except where noted.

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Introduction

Castilleja levisecta Greenm. (golden paintbrush) is listed as Endangered by the US Fish and Wildlife Service. The species once occupied prairies and grasslands throughout the Puget Trough and Willamette Valley. Habitat destruction and alteration over the past century have resulted in substantial declines in native vegetation in this ecoregion, and several species are now listed by state and federal agencies as threatened or endangered. All remaining populations of *C. levisecta* occur in Washington and British Columbia; the species is considered to be extirpated in Oregon.

Castilleja levisecta (Figure 1) is an herbaceous perennial that appears to reproduce only by seed. Like most paintbrushes (Heckard 1962), this species is a hemiparasite – its roots penetrate the roots of neighboring plant species and derive nutrients, carbohydrates, and possibly other secondary compounds from these hosts. The Recovery Plan for *C. levisecta* (USFWS 2000) identifies population reintroduction, development of propagation methods, and studies of the pollination biology of the species as high priority actions to meet recovery objectives.



Figure 1. *Castilleja levisecta* (left) and *C. hispida* (right).

A second paintbrush species, *C. hispida* Benth. (harsh paintbrush) (Figure 1), also occurs in prairies in this ecoregion. This species has been identified as a larval host plant for Taylor's checkerspot butterfly, another endangered species, and restoration with both paintbrush species is under consideration for improving butterfly habitat (Stinson 2005). There is concern, however, that plantings of the two species at the same site could lead to hybridization and alteration of the endangered *C. levisecta* genotype. To gauge this risk, an evaluation of the ability of these species to produce offspring from hybrid crosses is necessary. In this report, we present the results of controlled crosses between these species. In addition, we examine the geographic distribution of diploid and polyploidy forms in these species, and discuss the implications of our findings.

Goals and objectives

The goal of this study is to determine the potential for hybridization between *C. levisecta* and *C. hispida* in natural areas. The specific objectives are to:

- Determine experimentally whether hybrid crosses between *Castilleja levisecta* and *C. hispida* produce viable progeny as measured by seeds, plant growth and flowering, and pollen viability.
- Conduct a cytogeographic survey of *C. levisecta* and *C. hispida* to determine chromosome numbers in each and if ploidy level varies.

Methods

Fertility of hybrids

To assess the fertility of inter-species crosses between *Castilleja levisecta* and *C. hispida*, we grew plants of both species in a greenhouse and conducted experimental pollinations. Plants were grown from seed and co-planted with *Eriophyllum lanatum* as a host plant to maximize performance following Kaye (2003) (see cover photo). The seed source for *C. levisecta* was a wild population at West Beach (WB) on Whidbey Island, Island Co., Washington. Those for *C. hispida* were from populations at Fort Lewis, 13th Division Prairie (FL), Pierce Co., and Scatter Creek (SC), Thurston Co., Washington.

To germinate seeds, we placed them in cold stratification (5 C) on 12/19/06 then moved them to warm conditions in a greenhouse about six weeks later (on 1/26/07). We germinated 15 seeds and potted up to 5 seedlings from each of 10 maternal lines from each source on 3/1/07. Plants were fertilized weekly. Once plants began to flower, we performed various crosses within and between species using the different seed sources. Maternal lines were kept separate and each type of cross used each maternal line only once. We planned to produce ten crosses for each cross-type, but only eight were possible in many cases due to failure of some maternal lines. The types of crosses we performed are listed in Table 1. We balanced the crosses so that each cross type included roughly equal numbers of plants as the pollen donor (sire) and recipient (dam). Hand pollinations were performed April 21-23, 2007. Dams were not emasculated prior to pollination because previous research found *C. levisecta* to be self-sterile (Kaye 2003).

Table 1. Experimental crosses with *Castilleja levisecta* and *C. hispida*.

Cross type	Crossing design	Seed sources ¹	No. crosses
C. levisecta x C. hispida	C. l. (dam) x C. h. (sire)	Scatter Cr x West Beach	8
	C. h. (dam) x C. l. (sire)	West Beach x Scatter Cr.	8
		Subtotal	16
	C. l. (dam) x C. h. (sire)	Fort Lewis x West Beach	8
C. levisecta x C. levisecta	C. h. (dam) x C. l. (sire)	West Beach x Fort Lewis	9
		Subtotal	17
	C. l. (dam) x C. l. (sire)	West Beach x West Beach	10
		Subtotal	10
C. hispida x C. hispida	C. h. (dam) x C. h. (sire)	Scatter Cr. x Fort Lewis	8
	C. h. (dam) x C. h. (sire)	Fort Lewis x Scatter Cr.	8
		Subtotal	16
		Total crosses	68

¹Fort Lewis seed came from 13th Division Prairie.

Seed set: Pollinated flowers were marked with colored thread to track the cross-type, and plants were allowed to grow until 6/7/07 to mature their fruits. Once fully mature, the fruits were harvested and placed in paper packets. The total number of filled and unfilled seeds in each fruit were counted under a dissecting microscope and recorded.

Seed viability: Viability of these seeds was determined by germinating 30 seeds from each cross (Figure 2, left) following the same procedure as above to break dormancy and cause embryo growth. Germinated seedlings were counted two weeks after placement in warm conditions.



Figure 2. Left: preparing seeds from crosses for germination at the Oregon State University Seed Lab. Right: Plants from a variety of hybrid and within-species crosses on a greenhouse bench in a random layout.

Plant size: Up to five of the seedlings from each cross were potted in small pots. After 3 weeks of growth, three seedlings of each group of five were potted with a host (*Eriophyllum lanatum*) and grown in 4-inch pots in a greenhouse at Oregon State University. Seeds were germinated on January 2007, grown in the greenhouse to maturity (Figure 2, right), then measured on 4/21/08. Measurements on each plant included number of stems, total cumulative stem length, and number of flowers.

Pollen viability: Pollen viability was estimated from a limited sample size of individuals from each cross type. Staining of pollen grains is a common technique for evaluating pollen viability (Kearns and Inouye 1993). Pollen was extracted from the galea tip of a single flower with forceps and placed on a microscope slide with a drop of cotton blue stain, then covered with a glass cover slip. In this test, fertile pollen grains typically stain dark blue and sterile grains stain very light blue or not at all. Pollen grains were examined under a light microscope and scored as stained or not stained, and quality or intensity of staining was also noted. For each sample, between 300 and 369 pollen grains were examined.

Analysis: We used one-way ANOVA to test for crossing treatment effects on percentage seed set, seed viability, cumulative stem length, and flower number. Our preliminary analyses focused on keeping the different cross directions separate (i.e., considering the dam x sire vs. sire x dam as separate cross-types), but the direction of cross had no effect. Therefore, we combined all crosses within each cross-type in further analyses. Also, we used only data from crosses that successfully produced a fruit. We felt that those crosses that did not result in any fruit development were likely the result of our technique rather than cross incompatibility. Failure of fruit development occurred in 7 out of 68 crosses distributed across most cross types.

Chromosome number

Chromosome numbers were determined at various stages of meiosis in from pollen mother cells. Plants were grown from seed from a variety of sources in western Washington and British Columbia (Table 2, Figure 3) following propagation procedures as above. Once plants began to flower (April 2008), floral buds of appropriate size were removed and fixed in a mixture of 95% ethanol and glacial acetic acid (3:1 v:v) (Figure 4). Developing anthers with pollen cells undergoing meiosis were removed from the fixed buds, squashed in acetocarmine stain, and examined under a compound microscope for countable chromosome configurations.

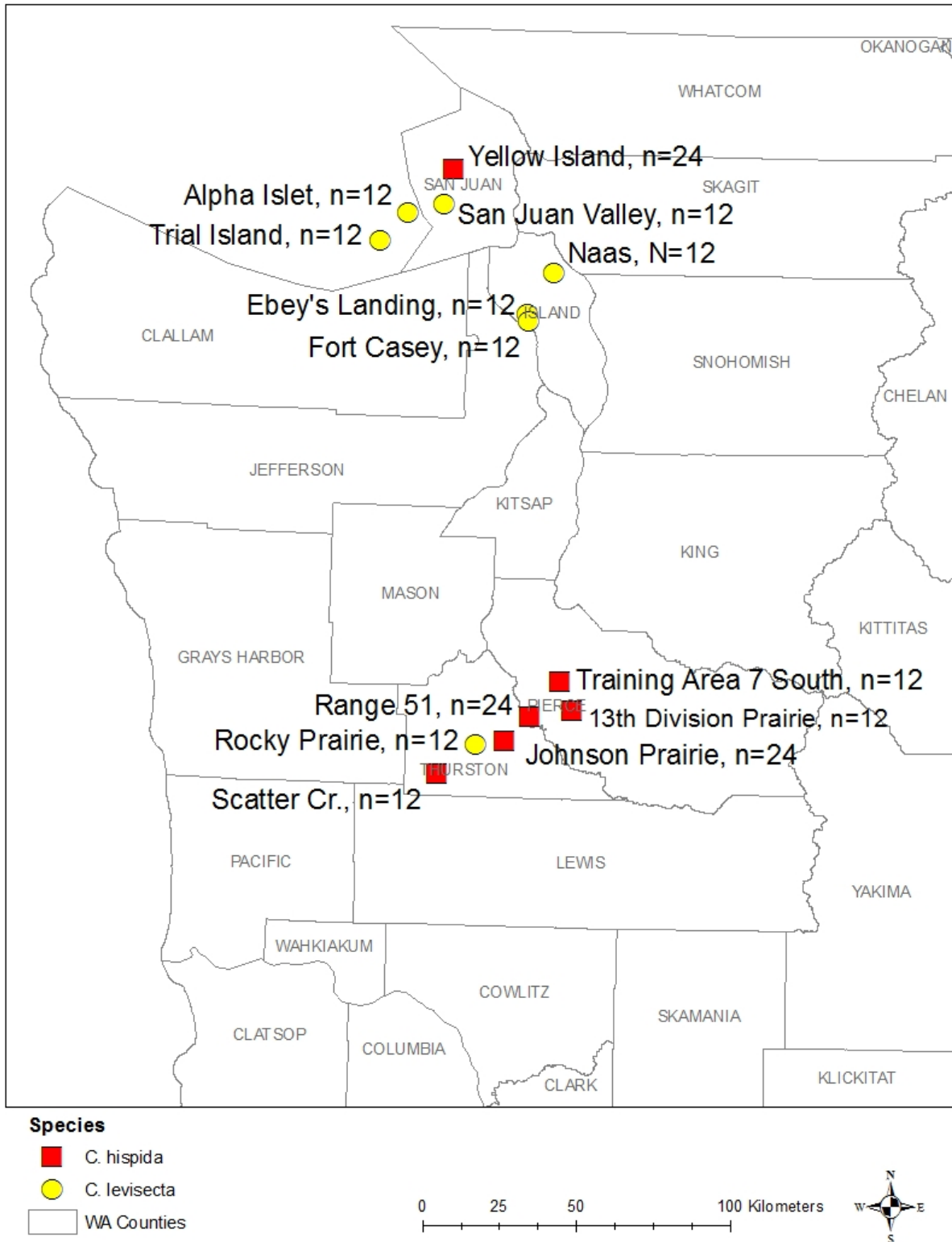


Figure 3. Geographic distribution of sample sites for chromosome counts. Seeds were collected from each site and grown in a greenhouse to produce plants for chromosome analysis.



Figure 4. Left: Gerry Carr samples bud tissue of *C. levisecta* and *C. hispida* in preparation for counting chromosomes. Right: *Castilleja* buds in fixative.

Results

Plant appearance

Within and between-species crosses generally produced plants that appeared healthy. Flowers of *Castilleja hispida* grown from a variety of sources for chromosome counts showed remarkable variation in flower size compared to *C. levisecta*. Some *C. hispida* plants, and all *C. levisecta* plants, had flowers around 2 cm long while the flowers on others were around 4 cm (Figure 4a). Hybrids appeared intermediate between parental types in coloring and morphology, although they tended to favor one parent or the other overall (Figure 5 b-c).

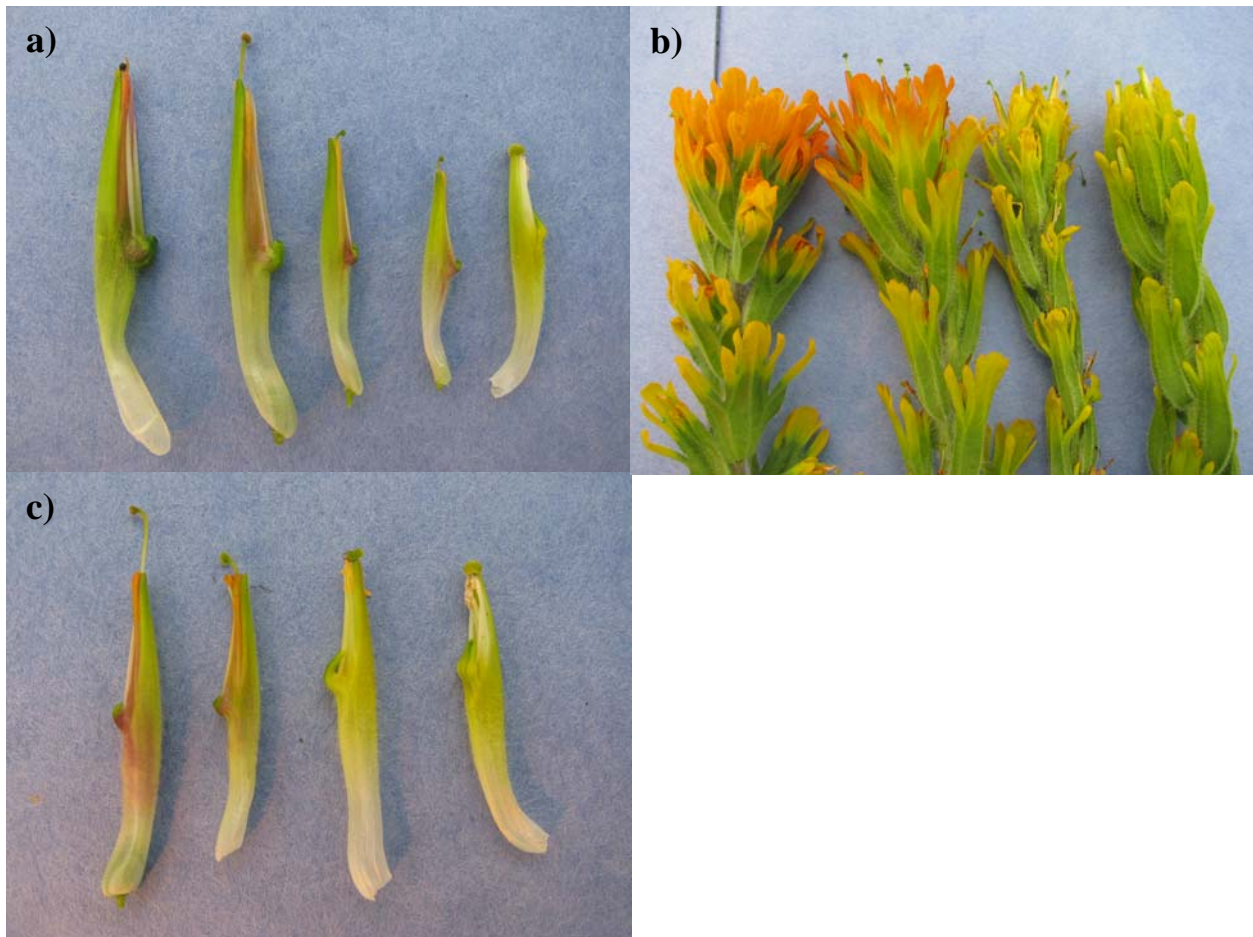


Figure 5. Flower morphology of *Castilleja levisecta*, *C. hispida* and their hybrids as grown in a greenhouse: a) left four flowers are *C. hispida* and the right flower is *C. levisecta*; b) left stem is *C. hispida*, center two are hybrids, and right stem is *C. levisecta*; c) left flower is *C. hispida*, center two are hybrids, and right flower is *C. levisecta*.

Fertility of hybrids

Cross-type had a significant effect on seed set ($p=0.0004$), and this effect was due to higher seed set from within *C. levisecta* crosses than within and between species crosses involving *C. hispida* (Figure 6a). Average seed set in same-species crosses with *C. levisecta* was 85% on average, but only 57% from *C. hispida* crosses. Hybrid crosses produced average seed set intermediate between these means but not significantly different from pure *C. hispida*. Seed set was 69% from *C. hispida* from Fort Lewis (13th Division Prairie) crossed with *C. levisecta*, and 63% from crosses between *C. hispida* from Scatter Creek and *C. levisecta*.

Seed germination was also affected by cross-type ($p<0.0001$) (Figure 6b). Within-species *C. levisecta* crosses yielded an average 96% germination, while *C. hispida* crosses resulted in only 49%. Germination from hybrid crosses was intermediate, but still relatively high at 80-90%.

Crossing had only a weak effect on cumulative stem length of plants grown in a greenhouse ($p=0.0802$) (Figure 6c). Hybrid plants were generally larger than parental types. *C. hispida* plants produced an average of 83 cm of stem and *C. levisecta* plants produced an average of 93 cm. Hybrids between *C. levisecta* and Fort Lewis *C. hispida* yielded plants with an average of 124 cm of stem production.

Flower production was strongly affected by cross-type ($p=0.004$) (Figure 6d). *Castilleja hispida* crosses yielded plants with 52 flowers on average, and *C. levisecta* plants produced 54 flowers. Hybrids between the two species produced plants with significantly more flowers, 89 to 91 on average, depending on the *C. hispida* seed source.

Pollen viability also appeared to be affected by cross type (Figure 7). Pollen staining suggested that pollen viability was substantially reduced in hybrid individuals compared to non-hybrid *C. levisecta* and wild forms of *C. hispida*. *C. levisecta* had 91% pollen stainability while average stainability from three wild sources (Yellow Island, Fort Lewis Range 51, and Fort Lewis 13th Division Prairie) of *C. hispida* was 74%. All of these had clear or fairly clear distinction of stained vs. unstained pollen grains. *C. hispida* plants resulting from same-species crosses averaged 46% pollen stainability with clear distinction between stained and unstained grains. Interspecific hybrids exhibited relatively low average pollen stainability of 23% and 44% with *C. hispida* parent sources of Scatter Creek and Fort Lewis, respectively. These samples, especially those from hybrids involving *C. hispida* plants from Scatter Creek, frequently had poor or very poor distinction between stained and unstained pollen, and light overall staining. Poor staining may indicate lack of pollen viability and therefore our estimates of viability are likely overestimates in these cases. In other words, pollen viability in the hybrids is likely even lower than our pollen stainabilities suggest.

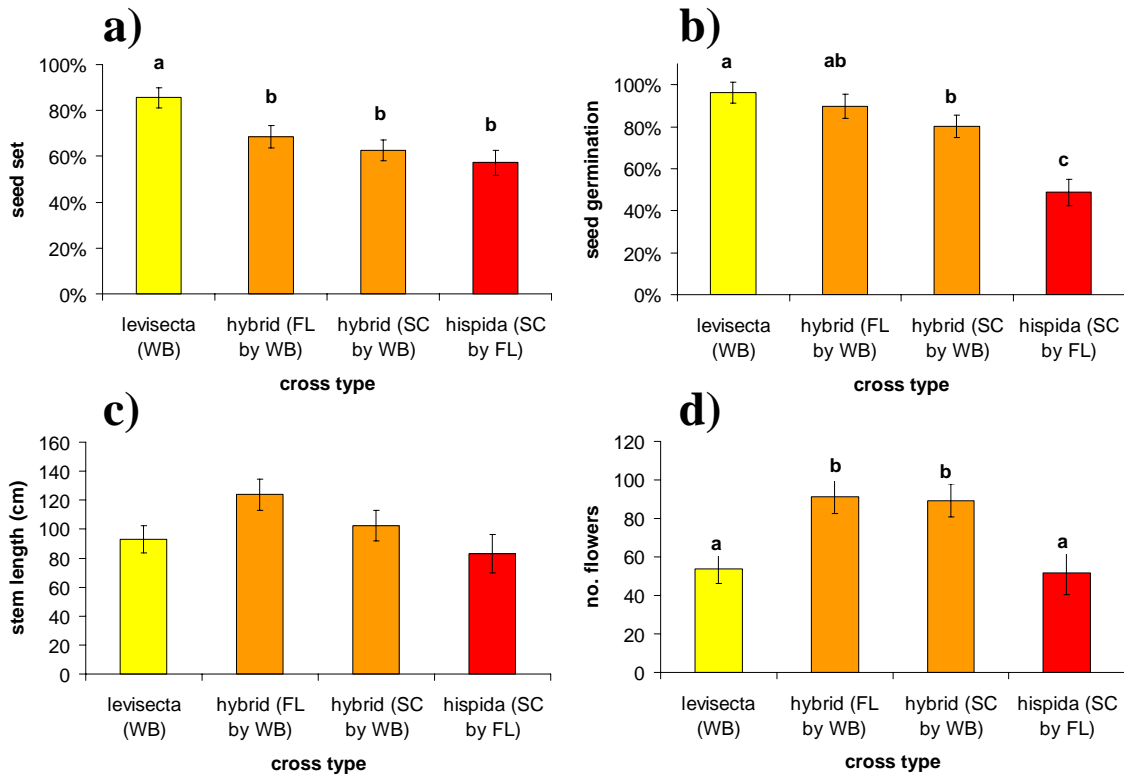


Figure 6. Effect of cross type on a) seed set, b) seed germination, c) cumulative stem length, and d) number of flowers per plant from within-species and hybrid crosses of *Castilleja levisecta* (WB=West Beach) and *C. hispida* (FL=Fort Lewis, SC=Scatter Creek). Bars with the same letter do not differ significantly ($p < 0.05$).

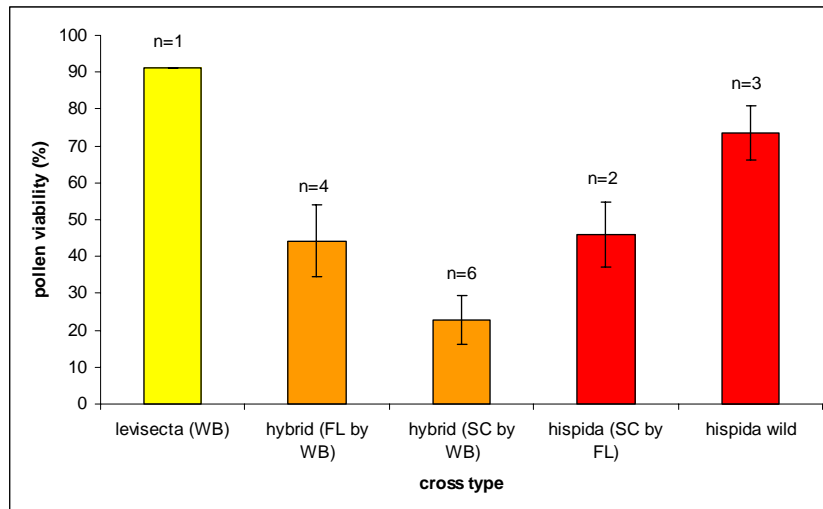


Figure 7. Pollen stainability (as an index of pollen fertility) from within-species and hybrid crosses of *Castilleja levisecta* (WB=West Beach) and *C. hispida* (FL=Fort Lewis, SC=Scatter Creek), as well as uncrossed, wild-type *C. hispida* plants from Fort Lewis (Range 51, 13th Division Prairie) and Yellow Island.

Chromosome number

Chromosome numbers were determined in individuals representing seven populations of *C. levisecta* and five populations of *C. hispida* (Table 2). Material suitable for chromosome number determination was not available for the Forbs Point, Washington population of *C. hispida*. Plants from Scatter Creek were inferred to be diploid ($n=12$) based on the diploid condition determined for hybrids involving this source. Chromosome numbers of $n = 12, 24,$ and 36 were found among six populations of *C. hispida* (Table 2). At least one population, Yellow Island, had both tetraploid and hexaploid plants present. To add additional perspective, it is important to note that the Yellow Island population is the only one for which more than one plant (3) was sampled. In contrast to the situation in *C. hispida*, individuals from all 7 populations of *C. levisecta* studied uniformly exhibited a diploid chromosome level ($n=12$) (Figure 8).

Table 2. Chromosome counts from various populations of *C. levisecta* and *C. hispida* in Washington.

Species	Site	n=
<i>C. hispida</i>	Johnson Prairie	24
	Range 51 AIA	24
	13th Div. Prairie	12
	Training Area Seven South	12
	Yellow Island	24, 36 [†]
	Scatter Creek	12 [‡]
<i>C. levisecta</i>	Ft. Casey	12
	Ebey's Landing	12
	NAS (Bocker)	12
	Trial Island	12
	Alpha Islet	12
	San Juan Valley	12
	Rocky Prairie	12 [¥]

[†] Two individuals exhibited $n=24$, a third exhibited $n=36$ with multivalents.

[‡] Chromosome number inferred from diploid interspecific hybrids involving parents from this population.

[¥] An unpublished count by Lawrence Heckard in 1988 also found $n=12$ at this site.

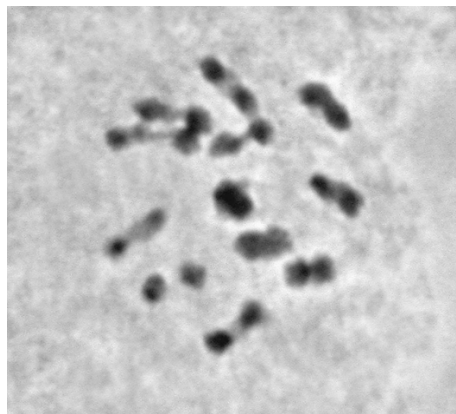


Figure 8. Diploid ($n=12$) chromosome condition of *Castilleja levisecta*. Photo courtesy of Gerry Carr.

Flower size and other plant traits in *C. hispida* differed with ploidy level. Diploid plants had smaller flowers and bracts than polyploids (Figure 9). On average, diploid flowers were ~23 mm long while those on polyploids were ~40 mm as measured from the flower base to tip of the galea. These features were consistent on greenhouse grown plants, but reports from our colleagues conducting field work suggest that polyploids in the wild may have somewhat shorter flowers, on the order of 30 mm.

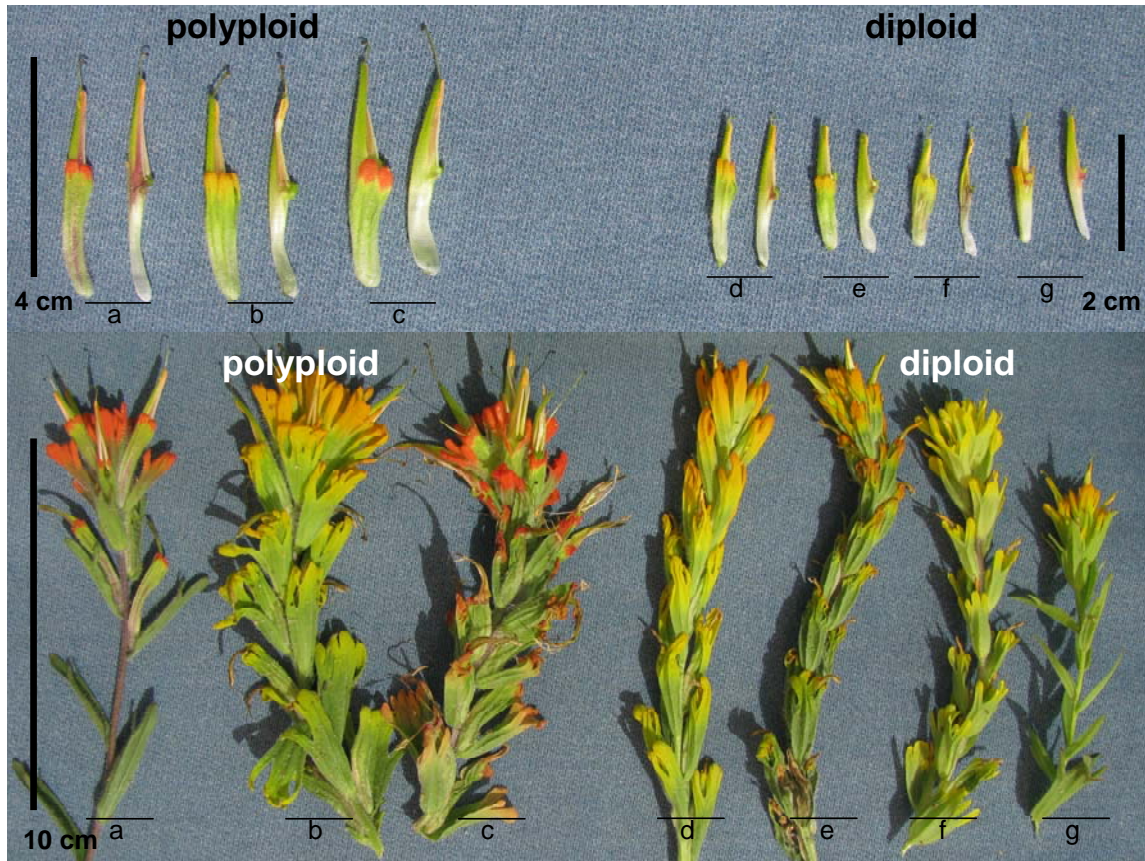


Figure 9.

Top: Floral morphotypes associated with polyploid (left) and diploid (right) forms of *Castilleja hispida* grown in a greenhouse; flowers of tetraploid plants were approximately 40 mm long, while diploid plants had flowers approximately 23 mm. Each pair for flowers is from one individual; left flowers have the calyx present and right flowers of have the calyx removed.

Bottom: Flowering stems of polyploid (left) and diploid (right) forms of *Castilleja hispida* grown in a greenhouse; note the greater degree to which flowers are exserted in polyploids.

Source locations for top and bottom: a) Yellow Island, b) Fort Lewis, Range 51, c) Yellow Island, d-e) Fort Lewis, 13th Division Prairie, f-g) Fort Lewis, Training Area 7 South.

Discussion

Our studies found no evidence of post-pollination reproductive barriers to gene flow between *C. hispida* and *C. levisecta* from seed set to germination, and growth through flower production in F1 hybrids (Figure 6). Pollen viability, however, appears to be reduced in hybrid individuals compared to parental forms (Figure 7), which may weaken the ability of hybrids to interbreed or form successful back crosses to their parental species.

Hybridization is possible between many species of *Castilleja* and is commonly reported among species in the field. Various authors have identified spontaneous hybrids between *Castilleja* species (e.g., Anderson and Taylor 1983), and gene exchange through hybridization appears to be an important process in the evolution of the genus (Heckard 1968, Chuang and Heckard 1991). Taxa in the Scrophulariaceae and in particular *Castilleja* have been found to have more spontaneous hybrids in the Intermountain west than might be expected due to chance (Ellstrand et al. 1996). Heckard (1968) suggested that interbreeding is possible not only between species with the same chromosome number but also between different species with different polyploid levels. Heckard and Chuang (1977) reviewed polyploidy and hybridization in 35 western *Castilleja* species and found examples of hybridization and intergradation between various species, but mostly where chromosome numbers were the same in each parent. These authors later reported that most artificial crosses between species, even from different genera, produced hybrids of varying fertility as long as both species had the same base chromosome number ($x=12$ for *Castilleja*) (Chuang and Heckard 1991). Previously reported chromosome numbers for *Castilleja hispida* include $n=12$ on the Oregon coast (Heckard 1968) and on Mary's Peak in the Coast Range (Chambers et al. 1998); $n=24$ in British Columbia north of Idaho, in Glacier National Park, and 12 miles east of Stevens Pass in Washington (Heckard and Chuang 1977); and $n=48$ for var. *acuta* in Wasco County, Oregon. We found $n=12$ and $n=24$ in plants from western Washington and the Gulf Islands of British Columbia, and at one site, Yellow Island, $n=24$ as well as $n=72$. No published accounts for *C. levisecta* are available, although Lawrence Heckard reported $n=12$ for plants from Rocky Prairie in his correspondence with Mark Egger. We found only diploid forms of *C. levisecta* in our evaluations.

Genetic swamping through hybridization has been identified as a threat to various rare plant species (Rieseberg 1991, Ellstrand 1992, Levin et al. 1996, Rhymer and Simberloff 1996), such as *Oenothera wolfii* (Imper 1995, Carlson and Meinke 2008). The degree to which hybridization creates a conservation risk for *C. levisecta* depends on the fitness and fertility of crossed progeny, and the likelihood that inter-specific hybridizations will occur in the wild. We have shown that *C. levisecta* and *C. hispida* can produce viable progeny, but that these hybrids have reduced pollen viability. Further, because *C. levisecta* is diploid, the risk of gene flow between these two species would likely be lowest at sites of sympatry involving polyploid forms of *C. hispida*. Polyploid *C. hispida* may be identified based on flower morphology and other traits. Thus, it may be possible to optimize conservation efforts through site selection and reintroduction strategies that limit contact between *C. levisecta* and diploid *C. hispida*, and where some degree of proximity is unavoidable, make sure that the *C. hispida* on site is polyploid.

Although hybrids between *C. levisecta* and *C. hispida* may be readily produced experimentally, they appear to be uncommon under field conditions. Both species are much less abundant in

prairies of western Washington than in the past. In 1984, the species co-occurred at Rocky Prairie and grew adjacent to one another (Figure 10), but no hybrid or morphologically intermediate individuals have been reported from that location, despite intense botanical attention at the site over the last two or more decades. Since 1984, *C. hispida* appears to have died out naturally at Rocky Prairie. Pollinator behavior may lead to few inter-specific crosses because of flower color differences. The yellow flowers of *C. levisecta* likely attract bumblebees and solitary bees, while the red flowers of most *C. hispida* may attract hummingbirds. Even if orange and yellow forms of *C. hispida*, such as plants from 13th Division Prairie at Fort Lewis, are planted near *C. levisecta*, pollinators may distinguish between the taxa based on other cues and concentrate their pollen foraging activities on one species at a time.



Figure 10. Sympatric individuals of *Castilleja levisecta* (right) and *C. hispida* (left) at Rocky Prairie preserve in 1984. Photo by TN Kaye.

Conclusions and Recommendations

The risk to *Castilleja levisecta* of genetic swamping from *C. hispida* appears to be relatively low and manageable. The following recommendations are intended to minimize the risk of hybridization during restoration activities for Taylor's checkerspot butterfly as well as recovery actions for *C. levisecta* at the same locations:

- Avoid inter-planting *C. levisecta* and *C. hispida*.
- Use polyploid *C. hispida* when planting near *C. levisecta*.
- If both species are planted at a nursery facility, keep them a minimum of 75 m apart.
- Focus further research on examining pollinator behavior in the field to determine if pollinators travel between experimental patches of *C. levisecta* and *C. hispida*.

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